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B. TABLE OF CONTENTS

| | <u>Page</u> |
|------------------------------|-------------|
| Cover | 1 |
| SF298 | 2 |
| Table of Contents | 3 |
| Introduction | 4 |
| Body | 4 |
| Key Research Accomplishments | 6 |
| Reportable Outcomes | 6 |
| Conclusions | 7 |
| References | 7 |
| Appendices | 7 |

INTRODUCTION

In this IDEA Award we proposed to test the hypothesis that the peripheral-type benzodiazepine receptor (PBR) may be used as a marker for the detection and diagnosis of breast tumors and that the regulation of PBR expression/function may eventually help in understanding the mechanisms involved in the progression of breast cancer and developing new tools for the treatment of the disease. Based on our initial preliminary studies on human breast cancer cells (1) we proposed two aims: (i) examine the expression and subcellular localization of PBR in a panel of human tumor breast biopsies and normal breast tissue biopsies and (ii) determine the function of PBR in human breast tumor cell lines. In this progress report we present preliminary data from the screening of the human biopsies and evidence on the role of PBR in human breast tumor cell growth.

BODY

In the approved statement of work we proposed two tasks:

1. Analyze the expression and subcellular localization of PBR in human tumor breast biopsies.
2. Determine the structure/function of PBR in human breast tumor cell lines.

1. Expression of Peripheral Benzodiazepine Receptor (PBR) in Human Tumors: Relationship to Breast, Colorectal and Prostate Tumor Progression (Appendix 1)

High levels of peripheral-type benzodiazepine receptor (PBR), the alternative-binding site for diazepam, are part of the aggressive human breast cancer cell phenotype *in vitro*. We examined PBR levels and distribution in normal tissue and tumors from multiple cancer types by immunohistochemistry. Among normal breast tissues, fibroadenomas, primary and metastatic adenocarcinomas, there is a progressive increase in PBR levels parallel to the invasive and metastatic ability of the tumor ($p < 0.0001$). In agreement with these protein data, breast tumors showed increased PBR mRNA levels compared with normal breast tissue. We then examined whether the correlation between PBR levels and aggressive tumor phenotype was restricted to breast tissue. In colorectal and prostate carcinomas, PBR levels were also higher in tumor than in the corresponding non-tumoral tissues and benign lesions ($p < 0.0001$). In contrast, PBR was highly concentrated in normal adrenal cortical cells and hepatocytes, whereas in adrenocortical tumors and hepatomas PBR levels were decreased. Moreover, malignant skin tumors showed decreased PBR expression compared with normal skin. These results indicate that elevated PBR expression is not a common feature of aggressive tumors, but rather may be limited to certain cancers, such as those of breast, colon-rectum and prostate tissues, where elevated PBR expression is associated with tumor progression. Thus, we propose that PBR overexpression could serve as a novel prognostic indicator of an aggressive phenotype in breast, colorectal and prostate cancers.

A manuscript describing in detail these findings has been submitted for publication (Appendix 1).

We are in the process of evaluating the subcellular distribution of the receptor and its relationship to the aggressive phenotype of the tissues.

2a. Peripheral-type Benzodiazepine Receptor Levels Correlate with the Ability of Human Breast Cancer MDA-MB-231 Cell Line to Grow in SCID Mice (Appendix 2)

MDA-MB-231 (MDA-231) human breast cancer cells have a high proliferation rate, lack the estrogen receptor, express the intermediate filament vimentin, the hyaluronan receptor CD44, and are able to form tumors in nude mice. The MDA-231 cell line has been used in our laboratory to examine the role of the peripheral-type benzodiazepine receptor (PBR) in the progression of cancer. During these studies two populations of MDA-231 cells were subcloned based on the levels of PBR. The subclones proliferated at approximately the same rate, lacked the estrogen receptor, expressed vimentin and CD44, and had the same *in vitro* chemoinvasive and chemotactic potential. Both restriction fragment length polymorphism and comparative genomic hybridization analyses of genomic DNA from these cells indicated that both subclones are of the same genetic lineage. However, only the subclone with high PBR levels was able to form tumors when injected in SCID mice. These data suggest that the ability of MDA-231 cells to form tumors *in vivo* may depend on the amount of PBR present in the cells.

A manuscript describing in detail these findings has been submitted for publication (Appendix 2).

2b. Peripheral-type Benzodiazepine Receptor (PBR) Gene Amplification in MDA-MB-231 Aggressive Breast Cancer Cells (Appendix 3)

Our previous studies using human breast cancer cell lines, and present studies using animal models and human tissue biopsies have suggested a close correlation between the expression of the peripheral-type benzodiazepine receptor (PBR) and the progression of breast cancer. Although we previously reported a dramatic increase in PBR mRNA levels parallel to the aggressive phenotype of the breast cancer cells (Hardwick et al., Cancer Research, 1999, 59:831-842), we examined the possibility that gene amplification may also be one of the reasons of increased PBR protein levels in aggressive breast tumor cells and tissue biopsies. We investigated the genetic status of the PBR gene in two human breast cancer cell lines: MDA-MB-231 cells, which are an aggressive breast cancer cell line that contains high levels of PBR, and MCF-7 cells, which are a non-aggressive cell line that contains low levels of PBR. Both DNA (Southern) blot and fluorescence *in situ* hybridization (FISH) analyses indicated that the PBR gene is amplified in MDA-MB-231 relative to MCF-7 cells. These unexpected data suggest that PBR gene amplification may be an important indicator of breast cancer progression.

A manuscript describing in detail these findings has been submitted for publication (Appendix 3).

2c. Transfection of exogenous PBR to non-aggressive human breast cancer cells alters their proliferation rate.

We have previously shown that the MCF-7 human breast cancer cell line contains extremely low levels of PBR (Hardwick et al., Cancer Research, 1999, 59:831-842). To further investigate the function of PBR in cell proliferation we transfected the MCF-7 derivative cell line MCF-7 Tet-Off (MCF-7 TO) (Clontech, La Jolla, CA) with the full-length human PBR cDNA.

For these studies, 8×10^5 MCF-7 TO cells were co-transfected with 40 μ g of either the pBI-EGFP vector alone or pBI-EGFP containing the human PBR cDNA and 2 μ g of the pTK-Hyg vector via electroporation (220V, 950 μ F). pBI-EGFP is a bi-directional expression vector from Clontech (La Jolla, CA) possessing both the enhanced green fluorescent protein cDNA and a multi-cloning sequence under the expression of a bi-directional minimal cytomegalovirus promoter. The pTK-Hyg vector (Clontech, La Jolla, CA) confers hygromycin resistance and acts as a negative selection marker in this study. After electroporation, cells were plated on 10 x 100mm polystyrene culture dishes in Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS) without antibiotics for two days at 37°C, 6% CO₂. The cells were then maintained in

DMEM, 10% FBS supplemented with 100 μ g/ml hygromycin and 1 μ g/ml doxycyclin (Dox). After several weeks, colonies over 2mm in size were isolated and amplified in DMEM, 10% FBS supplemented with 100 μ g/ml hygromycin and 1 μ g/ml Dox.

Transfection of PBR into the MCF-7 TO cell line, a cell line with low PBR expression (0.76pmol/mg protein), yielded a 10-fold higher expression of PBR that was completely abrogated with the repressor Dox. Induced expression of PBR (MIP1 cells), achieves maximal PBR ligand binding of 4.9pmol/mg protein. The ligand binding affinities are identical for both cell lines (1.2nM). Further, the resultant MIP1 cell line had a much greater proliferation rate than did the MCF-7 TO cell line, demonstrating the role of PBR in cell proliferation. As with ligand binding, cell proliferation returned to normal in the presence of the repressor Dox (Figure 1). Further characterization of these clones in tumor invasion and metastasis models is in progress.

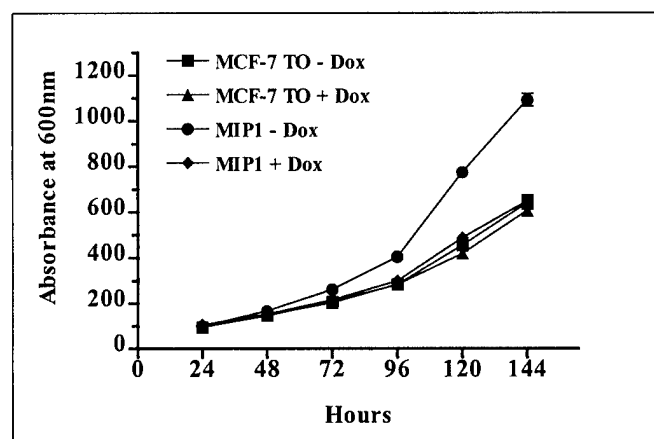


Figure 1. Cell Proliferation Curves for MCF-7 TO and MIP1 Cells. Six day growth curves were obtained for the MCF-7 TO and MIP1 cells in the presence and absence of 1.0 μ g/ml Dox. Cells were incubated at 37°C, 6% CO₂. Media with or without Dox was changed every 2 days. Cells were stained with Crystal Violet. The experiment was performed three times with eight replications for each cell line.

KEY RESEARCH ACCOMPLISHMENTS

We could summarize the following observations as the key research accomplishments of this funding period:

- Elevated PBR expression is not a common feature of aggressive tumors, but rather may be limited to certain cancers, such as those of breast, colon-rectum and prostate tissues, where elevated PBR expression is associated with tumor progression. Thus, we propose that PBR overexpression could serve as a novel prognostic indicator of an aggressive phenotype in breast, colorectal and prostate cancers.
- The ability of the aggressive, hormone-independent, MDA-MB-231 cells to form tumors *in vivo* may depend on the amount of PBR present in the cells.
- It appears that the increase in PBR expression in more aggressive cancers is the result of both gene amplification and increased gene transcription/translation.
- Expression of PBR in non-aggressive breast cancer tumor results in increased cell proliferation rates.

REPORTABLE OUTCOMES

- Hardwick M, Rone J, Han Z, Haddad B, Papadopoulos V (2001) Peripheral-type benzodiazepine receptor levels correlate with the ability of human breast cancer MDA-MB-231 cell line ability to grow in SCID mice. *International Journal of Cancer*, 94:322-327.

- Hardwick M, Rone J, Barlow K, Haddad B, Papadopoulos V (2002) Peripheral-type benzodiazepine receptor (PBR) gene amplification in MDA-231 aggressive breast cancer cells. *Cancer Genetics & Cytogenetics*, under revision.
- Han Z, Slack S R, Papadopoulos V (2002) Expression of peripheral benzodiazepine receptor (PBR) in human tumors: relationship to breast, colon and prostate tumor progression. Manuscript submitted.
- Han Z, Slack RS, Papadopoulos V (2002) Peripheral benzodiazepine receptor levels correlate with breast and other tumor progression and metastasis. Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting., Florida, USA.

CONCLUSIONS

In this progress report we present preliminary data from the screening of the human biopsies and evidence on the role of PBR in human breast tumor cell growth. These data indicate that aggressive, metastatic breast tumors contain higher levels of PBR compared to normal breast tissue. In these tumors PBR is preferentially localized around and in the nucleus. We also demonstrated that the ability of aggressive MDA-231 human breast cancer cells to form tumors *in vivo* correlates with the amount of PBR present in the cells. In an effort to characterize the breast tumor PBR, we uncovered two populations, the well-known 18 kDa protein and a 40-44 kDa fusion protein of PBR and nucleophosmin.

During the next funding period we will continue on with the screening of human breast cancer biopsies and the characterization of breast tumor PBR protein(s).

APPENDICES

1. Han Z, Slack S R, Papadopoulos V (2002) Expression of peripheral benzodiazepine receptor (PBR) in human tumors: relationship to breast, colon and prostate tumor progression. Manuscript submitted.
2. Hardwick M, Rone J, Han Z, Haddad B, Papadopoulos V (2001) Peripheral-type benzodiazepine receptor levels correlate with the ability of human breast cancer MDA-MB-231 cell line ability to grow in SCID mice. *International Journal of Cancer*, 94:322-327.
3. Hardwick M, Rone J, Barlow K, Haddad B, Papadopoulos V (2002) Peripheral-type benzodiazepine receptor (PBR) gene amplification in MDA-231 aggressive breast cancer cells. *Cancer Genetics & Cytogenetics*, under revision.

Expression of Peripheral Benzodiazepine Receptor (PBR) in Human Tumors: Relationship to Breast, Colorectal and Prostate Tumor Progression¹

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³The abbreviations used are: PBR, peripheral-type benzodiazepine receptor; GBM, glioblastoma multiforme; BPH, benign prostate hypertrophy

Abstract

High levels of peripheral-type benzodiazepine receptor (PBR), the alternative-binding site for diazepam, are part of the aggressive human breast cancer cell phenotype *in vitro*. We examined PBR levels and distribution in normal tissue and tumors from multiple cancer types by immunohistochemistry. Among normal breast tissues, fibroadenomas, primary and metastatic adenocarcinomas, there is a progressive increase in PBR levels parallel to the invasive and metastatic ability of the tumor ($p<0.0001$). In colorectal and prostate carcinomas, PBR levels were also higher in tumor than in the corresponding non-tumoral tissues and benign lesions ($p<0.0001$). In contrast, PBR was highly concentrated in normal adrenal cortical cells and hepatocytes, whereas in adrenocortical tumors and hepatomas PBR levels were decreased. Moreover, malignant skin tumors showed decreased PBR expression compared with normal skin. These results indicate that elevated PBR expression is not a common feature of aggressive tumors, but rather may be limited to certain cancers, such as those of breast, colon-rectum and prostate tissues, where elevated PBR expression is associated with tumor progression. Thus, we propose that PBR overexpression could serve as a novel prognostic indicator of an aggressive phenotype in breast, colorectal and prostate cancers.

Introduction

Two classes of benzodiazepine receptors have been identified, the central-type benzodiazepine receptor located on the neuronal plasma membrane, part of the GABA_A/benzodiazepine receptor complex (1) and the peripheral-type benzodiazepine receptor (PBR)³ (2). PBR was identified in peripheral tissues because of its ability to bind the benzodiazepine diazepam (valium™) (2). The pharmacological and molecular properties of these two receptors are distinct (2). PBR is an 18 kDa receptor protein which in steroid-synthesizing tissues, such as gonads, adrenal, placenta and brain, is extremely abundant. PBR primarily resides in the outer mitochondrial membrane where it regulates the transport of cholesterol to the mitochondrial inner membrane, the rate-determining step in steroidogenesis (2). Recent studies demonstrated that PBR is a high affinity cholesterol binding protein (3,4). PBR is also present in non-steroidogenic organs including kidney, lung, heart, liver and skin (2). In addition, it has been shown that PBR is involved in mitochondrial respiration (5), regulation of cell proliferation (6) and apoptosis (7).

PBR ligand binding, protein and mRNA levels were found to increase in a manner parallel to the increased aggressive phenotype of a battery of human breast tumor cells (8,9). PBR in aggressive MDA-MB-231 cells and human breast metastatic tumor biopsies is localized primarily in and around the nucleus, in contrast to the largely cytoplasmic localization seen in less aggressive MCF7 cells and in normal breast tissue (8). Moreover, the ability of MDA-MB-231 cells to form tumors *in vivo* depends on the amount of PBR present in the cells (10). In addition, drug-induced reduction of PBR levels in MDA-MB-231 cells was accompanied by reduced expression of several genes with close ties to either cell proliferation, differentiation, or apoptosis and correlated with reduced cell proliferation *in vitro* and tumor growth in nude mice (11).

Although there is evidence that PBR ligand binding capacity is higher in human tumors, such as

glioma, liver, colon, ovarian and endometrial carcinoma, than in the corresponding normal tissue (6,8,12-15), there is no indication that this increased PBR expression correlates with increased tumor progression and metastasis and no data on the expression of PBR in human breast, lung, skin, adrenal, testis and prostate tumors is available. In this report, we examined the expression of PBR in primary and metastatic human malignant tumor biopsies compared with corresponding normal tissue, benign lesions and with or without vicinal non-tumoral tissues. In addition, the correlation between PBR expression and tumor metastasis was evaluated. Our results show that elevated PBR expression is associated with breast, colorectal and prostate tumor progression.

Materials and Methods

Human biopsies. Human biopsies were obtained from Lombardi Cancer Center Tissue Resource at Georgetown University Medical Center, the Harvard Brain Tissue Resource Center (Belmont, MA) or ResGen (Huntsville, AL). Pathologists verified histological diagnosis and grading. Protocols for the use of human tissue were approved by the Georgetown University Internal Review Board. All samples were fixed in 10% formalin and embedded with paraffin. 5 μ m sections were cut and placed on glass slides.

Immunohistochemistry. All sections were deparaffinized and immunostaining was performed using an affinity-purified anti-peptide rabbit polyclonal anti-PBR antiserum raised against the conserved amino acid sequence 9-27 at a concentration of 1:400 (3). Immunoreactivity was detected using horseradish peroxidase-conjugated anti-rabbit IgG (1:500) (Transduction Laboratories, San Diego, CA). For the negative control, the primary antibody was replaced by 10% calf serum in PBS or preabsorbed with the PBR peptide used to generate the antiserum. Counterstaining was carried out with Mayer's hematoxylin (Sigma Diagnostics, St. Louis, MO).

Immunoreactivity was evaluated by staining intensity. PBR expression was evaluated by – (no staining or occasional cells stained), + (weak staining), ++ (moderate staining), +++ (strong staining) and ++++ (intense staining).

Semiquantitative RT-PCR. Pooled human normal brain, breast and colon tissue and corresponding tumor cDNAs were purchased from ResGen. PCR was carried out using the displayTAQ-Complete kit (Display System Biotech, Gaithersburgh, MD). Primers used for PCR amplification were as follows: (i) for PBR, sense 5'- ATGGGGTACGGCTCCTACCTGGTC and antisense 5'-TCACTCTGGCAGCCGCCGTCCCCC synthesized by Bio-Synthesis Inc. (Lewisville, TX) (ii) for human β -actin, used as a control, primers (sense 5'-TGACGGGGTCACCCACACTGTGCCCATCTA and antisense 5'-CATGAAGCATTTGCGGTGGACGATGGAGGG) were obtained from Stratagene (La Jolla, CA). In a 25 μ l volume, samples were denatured at 95° for 2 min (PBR) or 94° for 5 min (β -actin) and then subjected to 5 cycles of 95° for 20 sec, 65° for 15 sec and 72° for 1 min (PBR). Amplification was carried out for 19 cycles (PBR) or 18 cycles (β -actin) at 95° for 20 sec, 62° for 15 sec and 72° for 1 min (PBR) or 94° for 45 sec, 60° for 45 sec and 72° for 90 sec (β -actin) using a Bio-Rad iCycler (Hercules, CA). At the end of the cycles, the mixture was heated at 72° for 10 mins. PCR products were separated on 10% agarose gel containing 1 μ g/ml ethidium bromide. DNA bands were then scanned and analyzed by OptiQuant Acquisition and Analysis software (Packard Bioscience, Meriden, CT).

Statistical analysis. A pattern of increasing or decreasing PBR expression over increasing severity of histopathological types was tested within each organ using the exact Jonckheere-Terpstra test (16) as implemented in StatXact (17). The organs are breast, brain, colon-rectum, lung, ovary, prostate, skin, adrenal, liver and testis cancers. With 10 organs being tested, a

Bonferroni adjustment was used to control for multiple tests. PBR expression was considered to be significantly associated with histopathological severity if the p -value was less than 0.005 (0.05/10 organs). Since these are preliminary analyses, any organ with a p -value less than 0.05 was considered interesting for further investigation of this relationship.

Results and Discussion

We examined the expression of PBR in 10 types of human malignant tumor biopsies compared with corresponding non-tumoral tissues and benign lesions. PBR is constitutively present, at various expression levels, in all tissues examined (Fig. 1; Table 1). Both nuclear and cytoplasmic pattern of staining were observed.

Breast PBR expression levels (Figs. A1-A4) increased noticeably with increasing severity of breast lesion histopathology ($p < 0.0001$). Non-tumoral, fibroadenoma and adenosis cases were very similar with about 40% of cases with weak expression (+) and about 60% with moderate expression (++). Primary adenocarcinoma samples contained 3 (33%) cases with weak (+), and 4 (45%) with moderate expression (++) and 2 (22%) with strong staining (+++). The metastatic cases had only 9 (39%) with moderate staining and 14 (61%) with strong staining. These data show that higher levels of PBR expression are present in aggressive breast tumor cells. Non-tumoral tissue, fibroadenoma and adenosis tissues gave nearly identical results, with increasing expression in primary adenocarcinoma and even more in metastatic adenocarcinoma. These data are in agreement with *in vitro* studies on human breast cancer cell lines showing that PBR protein and drug ligand binding capacity were increased in aggressive breast cancer cells relative to non-aggressive cells (8,9). Moreover, these results also agree with data showing that the ability of aggressive breast tumor cells to form tumors *in vivo* might depend on the amount of

PBR present in the cells (10). Although in most cells PBR is primarily located in the mitochondria, a perinuclear/nuclear localization has been described in aggressive breast cancer and glioma cell lines (8,18). In the present study, both nuclear and cytoplasmic staining were observed. In agreement with these protein data and the *in vitro* cell data (8), breast tumors showed increased PBR mRNA levels compared with normal breast tissue (Fig. 2).

PBR expression in aggressive colorectal carcinomas (Figs. B1-B5) is significantly different from non-tumoral tissue and benign lesions ($p<0.0001$) and similar to that observed in breast cancer. All of the non-tumoral colorectal tissues presented only weak (+) PBR staining. Adenoma and primary adenocarcinoma samples showed 60% and 67% moderate (++) expression, respectively. Ten (47%) of the metastatic adenocarcinoma samples were intensely stained (++++), with only 2 (10%) with weak expression. These data are in agreement with earlier studies reporting increased PBR drug ligand binding sites in colon adenocarcinomas as compared to normal human colon (19). This increase of PBR in colorectal adenocarcinoma could be related to the role of benzodiazepines in relaxing smooth muscle contractions (19), which may cause slow passage of bowel contents with which colon cancer is associated. At the mRNA level (Fig. 2), however, the correlation does not appear to be so significant. One explanation is that there could be a difference of translational regulation of PBR expression between non-tumoral tissue and malignant carcinoma.

Brain tissue samples measured for PBR expression show a small trend indicating increased expression among GBM and astrocytomas (Fig. D1-D4). All three of the non-tumoral tissue samples showed weak PBR expression, while 9 (75%) of the GBM and astrocytoma samples had no staining (-), with 1 (8%) and 2 (17%) cases with weak (+) and moderate (++) expression, respectively. Immunoreactive PBR was found in the nuclei of the neurons and cytoplasm of glial

cells with weak strength in normal brain, whereas its expression increases in the group of GBM and astrocytoma. This trend is also consistent with the increased PBR mRNA levels in brain tumors (Fig. 2). These results suggest that PBR is expressed constitutively at low level until the onset of the tumor. It has been suggested that PBR expression is associated with the malignancy grade of astrocytoma and affect the life expectancy of tumor-bearing patients (6).

To the best of our knowledge, there has been no report about the correlation between normal lung and lung tumor PBR levels. All 10 normal lung tissues (Fig. E1) showed weak expression (+), while half (4 cases) of the adenocarcinomas had weak (+) and half moderate staining (++) (Fig. E2). This difference is significant ($p=0.02$) and although it suggests that PBR might be involved in the progression of lung cancer, further investigation is required.

There were no notable differences between the normal ovarian tissue and ovarian carcinomas ($p>0.99$), although this analysis was hampered by the availability of normal tissue samples (Fig. C1). The majority of tumor samples express PBR with 40% weak staining, 25% moderate staining and 20% strong and intense staining, respectively (Figs. C2 & C3). As the fourth leading cause of death in women, epithelial ovarian carcinoma is usually diagnosed in advanced stages. However, it has been reported that a robust increase in the number of PBR binding sites in ovarian carcinoma occurs when compared with benign ovarian tumors and normal tissues (14). Thus, it is still not clear whether PBR expression correlates with the progression of ovarian neoplasm.

PBR immunoreactivity was seen in all the prostatic tissues (Fig. J1) examined, but differed significantly between BPH samples and adenocarcinomas ($p<0.0001$). BPH cases (Fig. J2) exhibited mostly weak (+) staining (67%) with some moderate staining (++). Adenocarcinoma cases (Fig. J3) had moderate (++), 2 cases (13%), strong (+++), 8 cases (50%), and intense

(++++), 6 cases (37%), staining. These results indicate a strong correlation between PBR levels and prostate histopathology. Thus, PBR may be considered as one parameter along with prostatic specific antigen and prostate-specific acid phosphatase (20) in the diagnosis of prostatic adenocarcinoma. It has been reported that PBR drug ligand binding is present in both mitochondrial and microsomal fractions of Dunning G prostatic adenocarcinomas from orchiectomized rats compared to tumors from sham operated controls. Treatment with testosterone repressed PBR drug ligand binding in both fractions to control values, suggesting a role of testosterone on the PBR density in these hormone-sensitive prostatic tumors (21). Even though it is known that hormones regulate PBR levels, the molecular mechanisms involved in the regulation of PBR during tumor progression remain unknown. In contrast to the increased PBR levels observed in the epithelial tumors from breast, colon-rectum, prostate and lung, the opposite results were found in the tumors of skin, liver, adrenal and testis.

Normal skin tissue and less aggressive tumors have significantly ($p=0.002$) higher levels of staining compared to more advanced or malignant tissues (Fig. G1). Normal tissue showed PBR nuclear immunostaining ranging from weak (+, 66%), to moderate (++ , 17%) and to strong (+++, 17%) intensity, in agreement with a recent report (22), while basal cell carcinoma samples (Fig. G2) had mostly (86%) moderate (++) staining and the rest had weak (+) expression. Squamous cell carcinoma PBR immunostaining (Fig. G4) was mostly weak (+, 90%). Melanoma cases (Fig. G3) also presented mostly (86%) weak (+) staining. These data indicate that PBR is expressed at higher levels in the nucleus of skin epidermal cells whereas its expression level decreased in the corresponding tumors. It has been suggested that PBR may participate in an antioxidant pathway in normal skin epidermal cells (22).

Table 1 presents information about the levels of PBR expression present in adrenal (Fig. F1),

liver (Fig. H1) and testis (Fig. I1) tissues and their respective malignancies (Figs. F2, H2 & I2). The trend observed suggests that PBR is expressed at high levels in normal adrenal cortical cells, hepatocytes and Leydig cells as well as germ cells (although in reduced levels) of testis, whereas its presence decreased in their corresponding tumors accompanied with an exclusive nuclear staining in adrenal tumors and hepatocellular carcinomas. However, there were not enough samples to evaluate convincingly the statistical significance of these data. The present findings with liver tumors do not support the observation showing upregulation of PBR expression in human hepatocellular carcinoma (12).

In conclusion, among breast, colorectal and prostate carcinomas, PBR expression dramatically increases compared to their non-malignant counterparts. Metastatic breast and colorectal adenocarcinomas manifest increased PBR expression relative to their primary malignancies. Brain, lung and ovarian tumors show a small trend in which malignant tumors express more PBR than the corresponding non-tumoral tissues. We also observed that PBR is present in high levels in the cytoplasm of normal adrenocortical cells and hepatocytes, whereas PBR levels are lower in cortical adenoma, adenocarcinoma and hepatocellular carcinomas where it primarily localizes in the nuclei. PBR levels are higher in the epidermal cells of normal skin than in skin basal cell carcinomas, squamous cell carcinomas and melanomas. Testis seminomas show varied levels of PBR expression while the Leydig cells and germ cells contain PBR at very high and moderate levels, respectively. Despite the limited number of certain normal samples, these data provide a basis to study the predictive value of PBR expression in assessing the progression of the disease and the efficacy of various treatments. In addition, recent data showed the induction of apoptosis and cell death in various cancer cell lines by pharmacological concentrations of high affinity PBR drug ligands (8, 23-25). Taken together, these results support further efforts on the

evaluation of PBR as a predictive means of breast, colorectal and prostatic tumor progression and the use of these drugs as anti-tumor agents in clinical trials.

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Table 1. Comparison of PBR expression in human tumors and nonneoplastic tissue biopsies.

| Histopathological | | | PBR | | | | | |
|-------------------|---------------------------|-------|------------|------------|-------------|--------------|---------------|----------------|
| Tissue | Type | Cases | - N (%) | + N (%) | ++ N (%) | +++ N (%) | ++++ N (%) | <i>p</i> value |
| Breast | Non-tumoral tissue | 8 | - | 3(38) | 5(62) | - | - | <0.0001 |
| | Fibroadenoma, Adenosis | 22 | - | 9(41) | 13(59) | - | - | |
| | Primary adenocarcinoma | 9 | - | 3(33) | 4(45) | 2(22) | - | |
| | Metastatic adenocarcinoma | 23 | - | - | 9(39) | 14(61) | - | |
| Brain | Non-tumoral tissue | 3 | - | 3(100) | - | - | - | 0.25 |
| | GBM, Astrocytoma | 12 | - | 6(50) | 3(25) | 1(8) | 2(17) | |
| Colon | Non-tumoral tissue | 6 | - | 6(100) | - | - | - | <0.0001 |
| | Adenoma | 10 | 1(10) | 2(20) | 6(60) | 1(10) | - | |
| | Primary adenocarcinoma | 12 | - | 1(8) | 8(67) | 2(17) | 1(8) | |
| | Metastatic adenocarcinoma | 21 | - | 2(10) | 5(24) | 4(19) | 10(47) | |
| Lung | Non-tumoral tissue | 10 | - | 10(100) | - | - | - | 0.02 |
| | Adenocarcinoma | 8 | - | 4(50) | 4(50) | - | - | |
| Ovary | Normal tissue | 1 | - | 1(100) | - | - | - | >0.99 |
| | Adenocarcinoma | 20 | 3(15) | 8(40) | 5(25) | 3(15) | 1(5) | |
| Prostate | BPH | 15 | - | 10(67) | 5(33) | - | - | <0.0001 |
| | Adenocarcinoma | 16 | - | - | 2(13) | 8(50) | 6(37) | |
| Skin | Normal tissue | 6 | - | 4(66) | 1(17) | 1(17) | - | 0.002 |
| | Basal cell carcinoma | 7 | - | 1(14) | 6(86) | - | - | |
| | Squamous cell carcinoma | 10 | - | 9(90) | 1(10) | - | - | |
| | Melanoma | 7 | 1(14) | 6(86) | - | - | - | |
| Adrenal | Normal tissue | 1 | - | - | - | - | 1(100) | 0.40 |
| | Cortical adenoma | 3 | - | - | 3(100) | - | - | |
| | Cortical carcinoma | 1 | - | - | 1(100) | - | - | |
| Liver | Normal tissue | 1 | - | - | - | - | 1(100) | 0.57 |
| | Hepatocellular carcinoma | 13 | 3(22) | 1(8) | 4(31) | 1(8) | 4(31) | |
| Testis | Normal tissue | 1 | - | - | - | - | 1(100) | 0.11 |
| | Seminoma | 8 | 2(25) | 2(25) | 1(13) | 3(37) | - | |

PBR immunohistochemistry was carried out by incubating the tissue sections with affinity purified rabbit polyclonal anti-peptide anti-PBR antibody at a concentration of 1:400 overnight followed by incubating with HRP-conjugated rabbit IgG. PBR expression was evaluated by – (no staining or occasional cell stained), + (mild staining), ++ (moderate staining), +++ (strong staining) and ++++ (intense staining).

Figure Legends

Figure 1 PBR expression in human biopsies. PBR immunohistochemistry was carried out as described under *Materials and Methods*. PBR was highly expressed in primary (A3) and metastatic (A4) breast tumor compared with non-tumoral breast tissue (A1) and benign tumor (A2). The expression of PBR was increased in colorectal carcinoma (B3, B4 and B5) relative to non-tumoral tissue (B1) and adenoma (B2). Normal ovary (C1) showed very weak staining but ovarian carcinoma (C2 and C3) manifested increase of PBR expression. PBR positive cells were rare in normal brain tissue (D1) whereas the population of positive cells was markedly increased (D2, D3 and D4). Lung carcinoma cells (E2) expressed much more PBR than non-tumoral lung tissue (E1). PBR was highly expressed in the cytoplasm of normal adrenal (F1) and the nuclei of adrenal carcinoma tumor cells (F2). Normal epidermis (G1), basal cell carcinoma (G2), melanoma (G3) and squamous cell carcinoma (G4) all showed positive staining. Hepatocellular carcinoma (H1) showed decreased PBR Expression compared with its vicinal non-neoplastic hepatocytes and normal hepatocytes (H2). PBR was seen in the germ cells and Leydig cells of normal testis (I1) and seminoma tumor cells (I2). PBR expression was found increased in prostate adenocarcinoma (J3) relative to BPH (J2) and non-tumoral prostate tissue adjacent to BPH (J1). K shows an adrenal tumor biopsy where the primary antiserum was immunoneutralized by pre-incubation with the peptide used to generate the antiserum.

Figure 2. PBR mRNA levels in human brain, breast and colon tumors and corresponding normal tissues. RT-PCR methods were as described under in *Materials and Methods*. PBR mRNA levels are significantly increased in brain and breast tumors compared with their corresponding normal tissues. There is no significant difference in PBR mRNA levels between colon tumor and

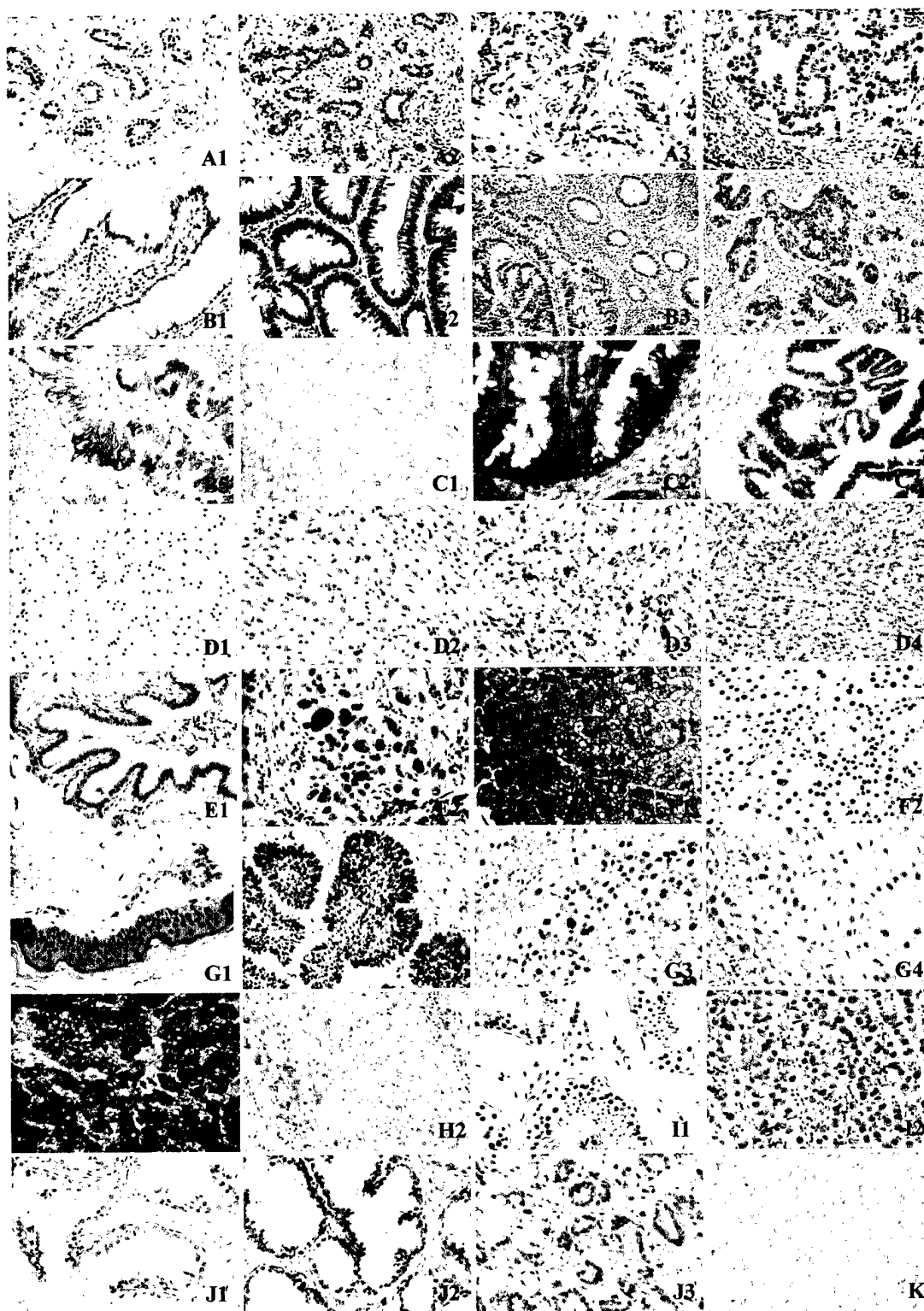


Figure 1

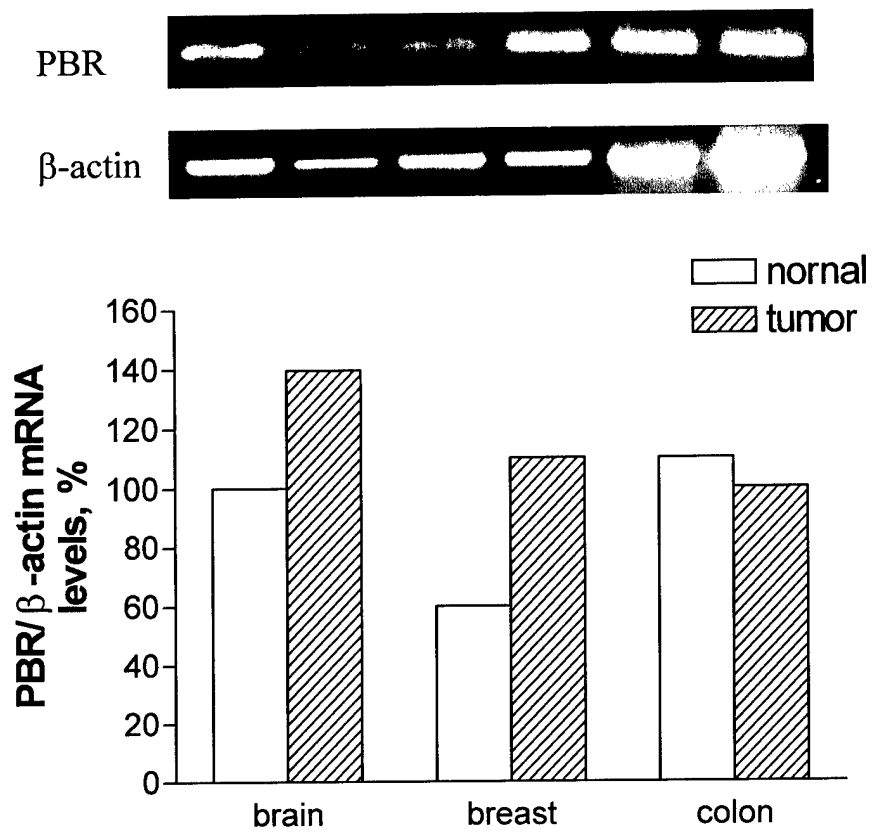


Figure 2

PERIPHERAL-TYPE BENZODIAZEPINE RECEPTOR LEVELS CORRELATE WITH THE ABILITY OF HUMAN BREAST CANCER MDA-MB-231 CELL LINE TO GROW IN SCID MICE

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MDA-MB-231 (MDA-231) human breast cancer cells have a high proliferation rate, lack the estrogen receptor, express the intermediate filament vimentin, the hyaluronan receptor CD44, and are able to form tumors in nude mice. The MDA-231 cell line has been used in our laboratory to examine the role of the peripheral-type benzodiazepine receptor (PBR) in the progression of cancer. During these studies 2 populations of MDA-231 cells were subcloned based on the levels of PBR. The subclones proliferated at approximately the same rate, lacked the estrogen receptor, expressed vimentin and CD44, and had the same *in vitro* chemoinvasive and chemotactic potential. Both restriction fragment length polymorphism and comparative genomic hybridization analyses of genomic DNA from these cells indicated that both subclones are of the same genetic lineage. Only the subclone with high PBR levels, however, was able to form tumors when injected in SCID mice. These data suggest that the ability of MDA-231 cells to form tumors *in vivo* may depend on the amount of PBR present in the cells.

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Key words: PBR; benzodiazepine; breast cancer; MDA-231

Aberrant cell proliferation and increased invasive and metastatic behavior are hallmarks of the advancement of breast cancer. In an effort to elucidate the mechanisms responsible for the progression of breast cancer, several laboratories have extensively characterized a battery of human breast cancer cell lines for several breast cancer markers such as the estrogen receptor (ER), the fibroblast cytoskeletal protein vimentin, the hyaluronan acid receptor, CD44, as well as their chemotactic and chemoinvasive potential.^{1–3} Although several studies have utilized these cell lines to determine the mechanism(s) responsible for the transition from non-aggressive to aggressive breast cancer, these mechanisms remain elusive. Work in our laboratory and others suggests that 1 potential candidate may be the peripheral-type benzodiazepine receptor (PBR). Recently we have shown that PBR expression is markedly increased in aggressive breast cancer cell lines, such as the MDA-231 cell line, relative to non-aggressive breast cancer cell lines as well as in aggressive metastatic human breast tumor biopsies compared with normal breast tissues.⁴ Moreover, ligands specific for PBR regulated the proliferation rate of the aggressive MDA-231 breast cancer cell line.⁴ PBR is an 18kDa transmembrane protein known to play a crucial role during steroidogenesis.^{5,6} Increased expression of PBR has been found in numerous tumor types and PBR has been shown to regulate the differentiation state and proliferation rates of several cancer cell lines.^{7–20} Over the past several years our laboratory has examined the role of PBR in breast cancer using the MDA-231 cell line. While examining MDA-231 cells from various origins we observed variations in their PBR levels. From the MDA-231 cell line that we previously used to investigate the role of PBR,⁴ subclones were isolated based on their PBR levels of expression. To determine if these differing PBR levels had any effect on the MDA-231 aggressive phenotype, we tested the anchorage-dependent and -independent proliferation of the subclones, as well as the expression of ER, vimentin, and

CD44. We also tested their *in vitro* chemotactic and chemoinvasive potential and their ability to grow in SCID mice.

MATERIAL AND METHODS

Cell culture

The MDA-231 cell line, obtained from the Lombardi Cancer Center, Georgetown University Medical Center, was grown as previously described.⁴ In some experiments cells were passaged twice in antibiotic-free DMEM, 10% FBS. Cells were then grown to confluency and maintained in without change of media for 5 days. All cultures were grown to 10 passages and either used or discarded. The media was then harvested and submitted to the Lombardi Cancer Center Tissue Core Facility for mycoplasma screening.

[³H]PK 11195 binding studies

Cells were grown to confluency in DMEM, 10% FBS. All cells were harvested from culture dishes and assayed for protein concentration by the Bradford method²¹ using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) and [³H]PK 11195 binding studies on 20 µg of protein from cell suspensions were performed as previously described.⁴ Scatchard plots were analyzed by the LIGAND program.²²

Crystal violet cell proliferation

Cells were grown to confluency, trypsinized, plated on 96-well plates at a concentration of 2,000 cells/well and maintained in DMEM, 10% FBS. Media was changed every 2 days. At the specified time points cells were stained Crystal Violet solution as described previously.¹⁷

Estrogen receptor enzyme immunoassay (ER-EIA)

Cells were harvested from confluent cultures by trypsinization and washed once with PBS. Cells were then washed once in phosphate buffer (5 mM sodium phosphate, pH 7.4, 10 mM thioglycerol, and 10% glycerol). Nuclear extracts from each cell line were prepared as previously described.²³ One hundred micrograms of each nuclear extract was incubated with 1 ER bead for 18 hr and processed according to the Abbott ER-EIA Monoclonal

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Protocol (1997). For each wash, beads were washed 10 times in distilled H₂O on a Brandel 24-well Harvester (Brandel, Gaithersburg, MD) in 12 × 75 mm borosilicate glass tubes.

Immunocytochemistry

Eight-chamber glass slides (Fisher Scientific, Pittsburgh, PA) were coated with poly-L lysine (Sigma Chemicals, St. Louis, MO) for several hr. Ten thousand cells per well for each cell line were loaded onto the slides and were incubated overnight at 37°C, 6% CO₂. The cells were fixed in 10% formaldehyde for 10 min at room temperature. After washing well with distilled H₂O, anti-PBR²⁴ diluted 1:400 in PBS supplemented with 10% FBS, anti-vimentin (Boehringer-Mannheim, Indianapolis, IN), diluted 1:3 in PBS supplemented with 10% FBS or anti-CD44 (Calbiochem, San Diego, CA) diluted 1:100 in PBS supplemented with 10% FBS, was added each well and incubated overnight at 4°C. After rinsing the slides well in PBS, horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (BD Biosciences Transduction Laboratory, Lexington, KY), diluted 1:1,000 in PBS supplemented with 10% FBS, was added to each well and incubated for 1 hr at RT. Visualization of HRP activity with 3-amino-9-ethyl carbazole (AEC) and counterstaining with hematoxylin was performed as previously described.⁴

Chemotactic and chemoinvasion assays

For chemotaxis assays, cells were grown to confluency, trypsinized, and plated on 8 µm 24-well inserts (Becton Dickinson Labware, Bedford, MA) at a concentration of 3 × 10⁵ cells/insert in DMEM, 0.1% FBS. The inserts were then placed in 24-well companion plates (Becton Dickinson Labware) containing 800 µl DMEM, 10% Tet-approved FBS (Clontech, San Diego, CA) and incubated at 37°C, 6% CO₂ for 48 hr. Before beginning the chemoinvasion assay, 8 µm 24-well inserts (Becton Dickinson Labware) were coated with 200 µg/cm² matrigel (Becton Dickinson Labware) and left to dry overnight. Several hr before the beginning of the assay, the matrigel was rehydrated with DMEM, 0.1% FBS. All cells were grown to confluency, trypsinized, and plated on matrigel-coated 8 µm 24-well inserts at a concentration of 3 × 10⁵ cells/insert in DMEM, 0.1% FBS. The inserts were then placed in 24-well companion plates (Becton Dickinson Labware) containing 800 µl DMEM, 10% FBS and incubated at 37°C, 6% CO₂ for 48 hr.

At the end of each incubation, cells were removed from the inside chamber by use of suction followed by a cotton swab. The membranes were then incubated for 10 min with Crystal Violet (0.5% Crystal Violet, 25% methanol) at room temperature. Excess Crystal Violet was removed by washing the membranes 6 times with room temperature tap H₂O. All staining of the inside of the membranes was removed with a cotton swab. Membranes were then dried overnight. The stain was solubilized from membranes and absorbance was read at 570 nm as previously described.¹⁷

Tumor growth in SCID mice

Cells were grown to confluency, trypsinized, counted and resuspended in sterile PBS at a concentration of 5 × 10⁵ cells/100 µl. One hundred micrograms of the cell suspensions were then injected into the mammary fat pad of 8 SCID mice for each cell

line. Tumor growth was measured weekly for 1 month. Protocols were approved by the Georgetown University Animal Care and Use Committee.

Restriction fragment length polymorphism (RFLP)

To examine the genetic identity of the MDA-231 PL and PH cells, frozen cell pellets were sent to Cellmark Diagnostics, Inc. (Germantown, MD). Genomic DNA (DNAGEN) was prepared, digested with HinfI enzyme, electrophoresed, blotted onto nylon membranes and hybridized with the indicated probes. Probe-DNA complexes were visualized by chemiluminescence (2000 Standard Operating Procedures, 100-25, Cellmark Diagnostics, Inc.).

Comparative genomic hybridization (CGH)

CGH was performed according to standard procedures.²⁵ Normal control DNA was prepared from peripheral blood lymphocytes of a cytogenetically normal male and tumor DNA was extracted from the 2 cell lines, MDA-231 PH and PL. Nick translation was performed to label tumor DNA with bio-16-dUTP (Boehringer-Mannheim, Germany) and control DNA with digoxigenin-11-dUTP (Boehringer-Mannheim). Each of the labeled genomes (500 ng) were hybridized in the presence of excess Cot-1 DNA (50 µg) (GIBCO-BRL, Gaithersburg, MD) to metaphase chromosomes prepared from a karyotypically normal donor. The biotin-labeled tumor genome was visualized with avidin conjugated to fluorescein isothiocyanate (FITC) (Vector Laboratories Inc., Burlingame, CA) and the digoxigenin-labeled control DNA was detected with a mouse anti-digoxin antibody (Sigma Aldrich) followed by detection with a goat anti-mouse antibody conjugated to tetra-methyl-rhodamine isothiocyanate (TRITC) (Sigma Aldrich). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and embedded in anti-fading agent to reduce photobleaching.

Gray scale images of the FITC-labeled tumor DNA, the TRITC labeled control DNA and the DAPI counterstain from at least 8 metaphases from each hybridization were acquired with a cooled charge-coupled device CCD camera (CH250, Photometrics, Tucson, AZ) connected to a Leica DMRBE microscope equipped with fluorochrome specific optical filters TR1, TR2, TR3 (Chroma Technology, Brattleboro, VT). Quantitative evaluation of the hybridization was done using commercially available software (Applied Imaging, Pittsburgh, PA). Average ratio profiles were computed as the mean value of at least 8 ratio images to identify chromosomal copy number changes in all cases.

RESULTS

We previously described the MDA-231 cell line as having a maximal PBR ligand binding (B_{max}) of 8.7 pmol/mg of protein.⁴ From this MDA-231 cell line we isolated sub-populations based on PBR levels. Scatchard analysis of the saturation isotherms generated using 2 of the isolated MDA-231 subclones indicated that these cells had divergent PBR ligand binding (Table I). Three of the new isolated cell lines had a B_{max} of 2.9 pmol/mg protein (Fig. 1a; Table I). Although all of the experiments reported herein were performed with all 3 cell lines, because the results obtained were similar only representative data from 1 of the cell lines are shown.

TABLE I—COMPARISON OF MDA-231 SUBCLONES FOR EXPRESSION OF VARIOUS MARKERS OF BREAST TUMOR AGGRESSION¹

| Cells | PK 11195 binding | | ER | Vimentin | CD44 | Chemoinvasion | Chemotaxis |
|------------|------------------|---------------------------------|----|----------|------|---------------|------------|
| | KD (nM) | B _{max} (pmol/mg prot) | | | | | |
| MDA-231 PH | 7.5 ± 0.9 | 6.4 ± 1.0 | — | + | + | +++++ | +++++ |
| MDA-231 PL | 1.8 ± 1.0 | 2.9 ± 1.0 | — | + | + | +++++ | +++++ |

¹PK 11195 binding values for the MDA-231 PH and PL cell lines were obtained from the Scatchard analyses presented in Figure 1. KD, dissociation constant; B_{max}, maximal PK 11195 binding. Quantification of estrogen receptor (ER) expression was determined using the ER-Enzyme Immuno-Assay (ER-EIA). —, the absence of ER expression. Vimentin and CD44 expression were determined by immunocytochemistry. +, visible detection of protein expression. Chemotaxis and chemoinvasion were determined as described in Material and Methods. +++++, values are 80–100% of MDA-231 PH values.

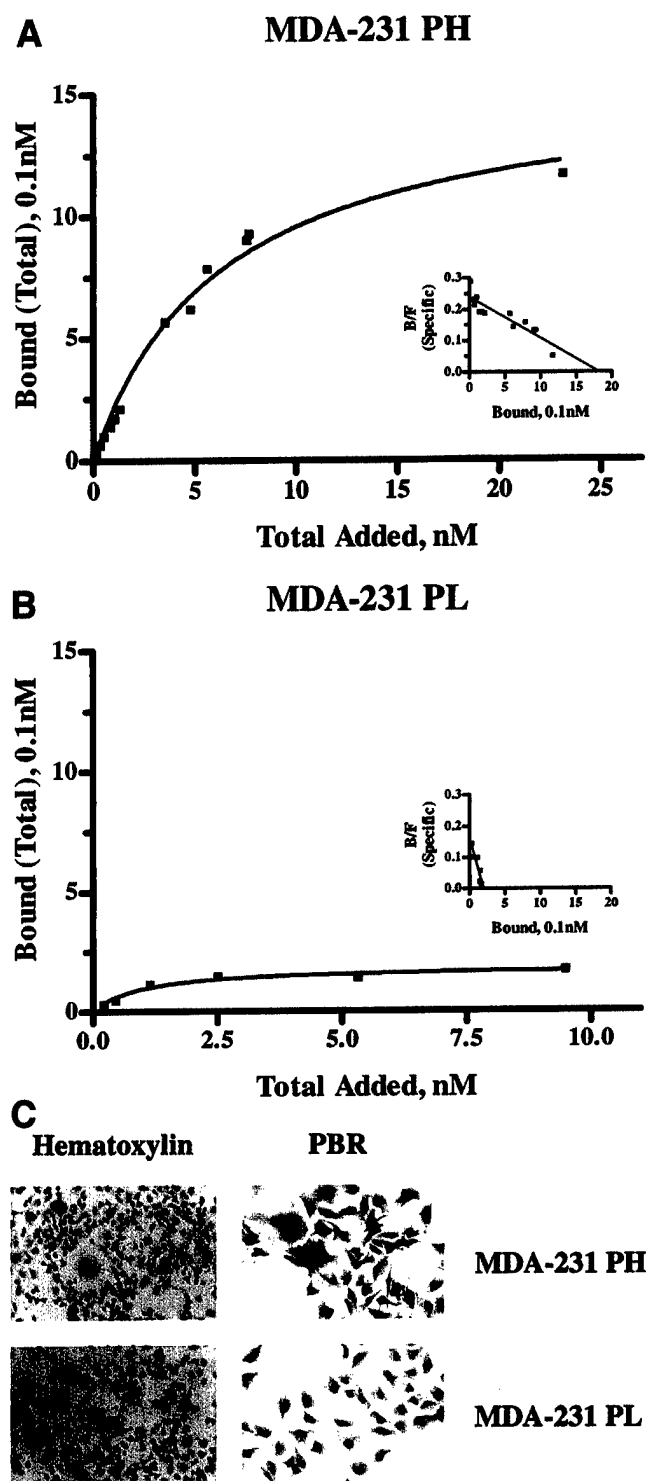


FIGURE 1 – PBR ligand binding characteristics and PBR immunoreactivity in the MDA-231 PH and MDA-231 PL cell lines. (a) PK 11195 ligand binding was measured in the presence of 20 μ g of cellular protein and the indicated concentrations of [3 H]PK 11195. Representative saturation isotherms and Scatchard plots are shown. (b) MDA-231 PH and MDA-231 PL were plated on slides, fixed, and stained either with hematoxylin or incubated with anti-PBR antiserum. Pictures were taken at 10 \times and 40 \times magnification for hematoxylin and PBR, respectively.

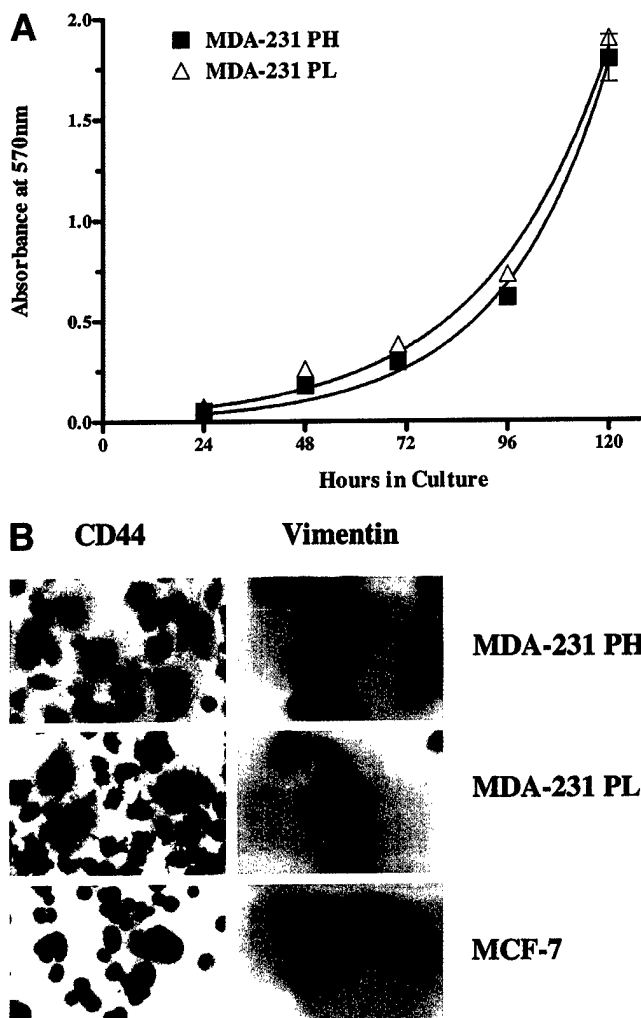


FIGURE 2 – MDA-231 PH and MDA-231 PL cell proliferation rates and expression of CD44 and vimentin. (a) MDA-231 PH and MDA-231 PL Cell proliferation rates. Cells were plated at a density of 2,000 cells per well on 96-well plates and followed for 6 days. Every 24 hr, cells were stained with Crystal Violet. Absorbance was read at 570 nm. Results shown are representative of 3–4 independent experiments. (b) MDA-231 PH and MDA-231 PL were plated on slides, fixed, and incubated with anti-vimentin or anti-CD44 antisera. Original magnification $\times 40$.

These cells are referred to MDA-231 PBR Low (MDA-231 PL). Other subclones of MDA-231 cells had a B_{max} equal or higher than 6.4 pmol/mg protein, similar to our original published results (Fig. 1a; Table I). Although all of the experiments reported herein were performed with 3 clones, because the results obtained were similar only representative data from 1 of the cell lines are shown. This cell line is called MDA-231 PBR High (MDA-231 PH). The MDA-231 PL cell line also differed from the MDA-231 PH cell line in the PK 11195 dissociation constant (K_D ; Fig. 1a; Table I). It should be noted that PBR ligand binding was performed multiple times over the course of several passages (approximately 10) and similar results were obtained. Differences in PBR expression seen with radioligand binding assays were confirmed with immunocytochemistry using the recently developed anti-PBR peptide affinity-purified antiserum (Fig. 1b).²⁴ Despite differences in B_{max} and K_D , the cells for all of the sub-populations appear morphologically similar (Fig. 1b).

Under normal culture conditions, both sub-populations appeared to grow at the same rate. To insure that this was the case, however,

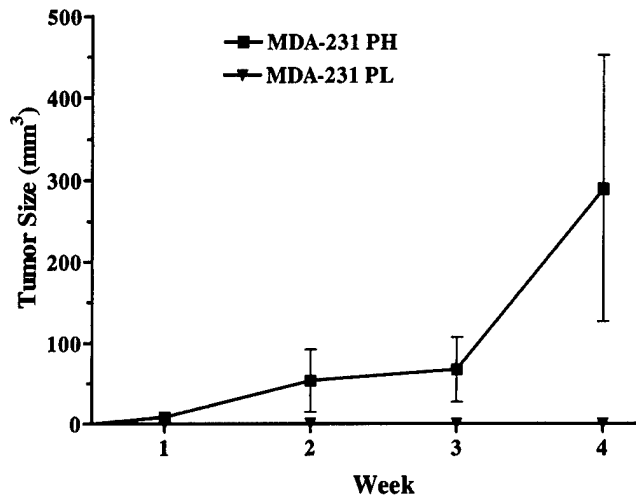


FIGURE 3 – MDA-231 PH and MDA-231 PL tumor growth in SCID mice. Cells were grown to confluency, trypsinized, counted and resuspended at a concentration of 5×10^5 cells/100 μ l in sterile PBS. One hundred μ l of cell suspensions was injected into each of 8 mice per cell line. Tumor growth was followed for 1 month with caliper measurements at the indicated times. Both cell lines tested negative for mycoplasma contamination (data not shown).

we followed the growth of each population over several days and quantified the number of cells with Crystal Violet (Fig. 2a). Over 5 consecutive days there was no appreciable difference between the MDA-231 PH and MDA-231 PL proliferation curves.

Our main hypothesis with regards to PBR in cancer is that increased expression of PBR confers a more aggressive phenotype in cancer cells. Therefore, decreased expression of PBR should indicate a differentiation toward a less-aggressive phenotype. As indicated above, the MDA-231 and several other breast cancer cell lines are well characterized for several breast cancer markers, as well as their chemotactic and chemoinvasive potential.¹⁻³ The MDA-231 cell line, as well as other late stage or highly-aggressive breast cancer cell lines, lack ER expression, express vimentin and CD44, and are highly chemotactic and chemoinvasive.¹⁻³ Conversely, early stage or non-aggressive breast cancer cell lines are ER positive, vimentin negative, express trace CD44 levels, and possess very little chemotactic or chemoinvasive potential.¹⁻³ We found no difference in the expression of ER (Table I), vimentin (Fig. 2b), or CD44 (Fig. 2b) between the MDA-231 sub-populations. Likewise, no significant differences in the chemotactic or chemoinvasive potential were found between MDA-231 PH and MDA-231 PL cells (Table I).

We then injected MDA-231 PH and MDA-231 PL cells into SCID mice to determine if their differences in PBR expression *in vitro* lead to alterations in their ability to grow *in vivo*. Not surprisingly, MDA-231 PH cells grew well in SCID mice (Fig. 3). In fact, 1 month post-injection several of the 8 MDA-231 PH-injected animals were put down due to too large tumors. The MDA-231 PL cells, however, did not form any detectable tumors in the mice (Fig. 3). To determine if this lack of growth was due to mycoplasma contamination, we submitted the cells to mycoplasma screening. Both the MDA-231 PH and the MDA-231 PL

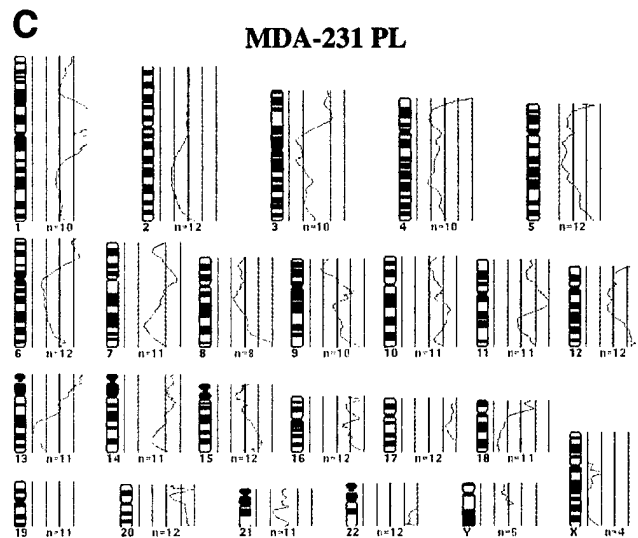
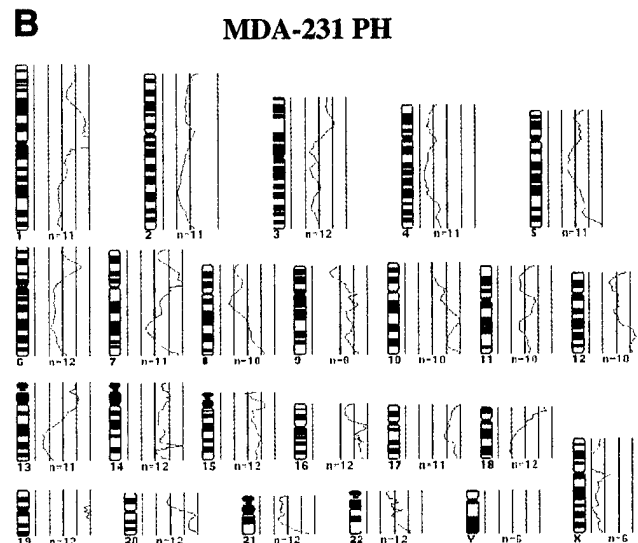
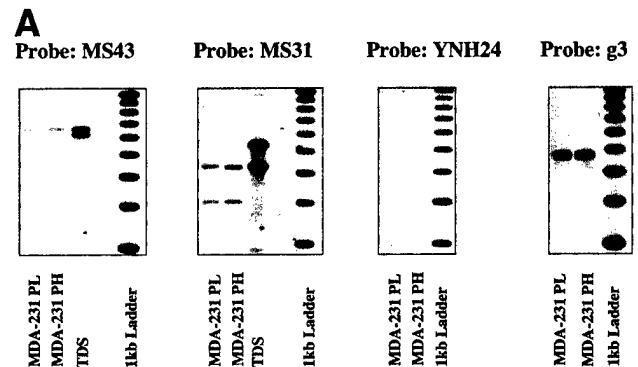


FIGURE 4 – RFLP and CGH DNA fingerprinting indicate that MDA-231 PH and MDA-231 PL are of the same genetic origin. DNA_{GEN} was isolated from both the MDA-231 PH and the MDA-231 PL cell lines and subjected to DNA fingerprinting analysis. (a) RFLP analysis of the indicated 4 independent chromosomal locations with the *Hinf*I restriction enzyme was performed on both samples. TDS, liquid blood human DNA control extracted and treated as were the samples under investigation. CGH analysis was performed on chromosomes 1 through 22 of both samples. DNA from the peripheral blood lymphocytes of a cytogenetically normal female was used as the normal control. Results of CGH evaluation of MDA-231 PL (b) and MDA-231 PH (c) showing a very similar pattern of chromosomal gains and losses. The 5 vertical lines on the right side of the chromosome ideograms reflect different values of the fluorescence ratio between the test and the normal DNA. The values are 0.5, 0.75, 1, 1.25 and 1.5 from left to right. Ratios of 1.25 or higher reflect gains whereas ratios of 0.75 or lower reflect losses. The ratio profile (curve) was computed as a mean value of 8 metaphase spreads (n is the number of chromosomes used to generate each ratio profile).

cell lines were negative for mycoplasma contamination (data not shown).

Given the dramatic differences in the *in vivo* growth of these MDA-231 sub-populations, and to determine if the MDA-231 PH and MDA-231 PL cell lines were derived from the same genetic lineage, we performed DNA fingerprinting using restriction fragment length polymorphisms (RFLP) on both cell lines and compared their chromosomal aberration using comparative genomic hybridization (CGH). Both the RFLP, as evaluated and interpreted by Cellmark Diagnostics Inc., and CGH determined that, indeed, the MDA-231 PH and MDA-231 PL are derived from the same genetic lineage and have similar chromosomal abnormalities (Fig. 4).

DISCUSSION

The MDA-231 human breast cancer cell line is widely used as an *in vitro* model for aggressive breast cancer. Our laboratory has used it to help elucidate the role of PBR in human breast cancer.⁴ During the course of these studies we observed variability in PBR levels of MDA-231 cells obtained from various sources. Using the MDA-231 cell line that we previously used to investigate the role of PBR in cancer progression and aggressive behavior of the cells we isolated 2 sub-populations differing in their expression of PBR ligand binding. The MDA-231 PH and MDA-231 PL cell lines do not differ in expression of the breast cancer markers ER, vimentin, or CD44. Similarly, these cell lines do not differ in their chemotactic or chemoinvasive potentials. They differ markedly, however, in their ability to grow in SCID mice. MDA-231 PH cells grow quite well in SCID mice, whereas MDA-231 PL cells do not.

CGH and RFLP data demonstrated that both subclones are derived from the same genetic lineage suggesting that the MDA-231 human breast cancer cell line is composed of an heterogeneous population. It is difficult to determine whether the differences in PBR expression between the 2 cell lines is due to innate properties of this heterogeneous population or to traits acquired over several passages. In our experience, the MDA-231 cell line has always been a morphologically heterogeneous population consisting of very large, round, flat cells intermixed with thin elongated cells. Both the MDA-231 PH and MDA-231 PL cell lines are morphologically similar, with no difference in the ratio of 1 cell type to another. Therefore, it is unlikely that the reduction of PBR expres-

sion seen in the MDA-231 PL cell line is due to the predominance of 1 cell morphology relative to the other. The data suggests that a reduction of PBR expression may occur over time as the result of multiple passages, however this hypothesis needs to be thoroughly tested before any conclusions may be drawn.

How does the reduction of PBR expression in the MDA-231 PL lead to the inability of these cells to grow in SCID mice? Our laboratory, as well as others, has previously shown that PBR ligands regulate cell proliferation.^{7-11,13-17} The *in vivo* behavior of the cells, however, differs from their *in vitro* behavior where no differences in the rates of cell proliferation between the MDA-231 PH and PL is seen. This may be due to a minimum of PBR levels required for cell proliferation. In our previous study, *in vitro* changes were seen with cells, such as the MCF-7 human breast cancer cells, which contain PBR levels 10-fold lower than the MDA-231 PL cells. This hypothesis is also supported by recent evidence that introduction of PBR into the MCF-7 cell line induces increases in the proliferation rate of these cells (Hardwick and Papadopoulos, unpublished data).

A close correlation between the reduction of MDA-231 cell proliferation and tumor growth in nude mice with PBR expression induced by the treatment of the cells and animals with the standardized *Ginkgo Biloba* extract EGb 761 was also shown.²⁶ Concomitant with the downregulation of PBR in MDA-231 cells by this treatment is the altered expression of several genes with close ties to either cell proliferation, differentiation, or apoptosis.²⁶ The majority of the genes affected by EGb 761 are members of either the Myc family of transcription factors and their effectors, the GTP-binding protein RhoA and its effectors, and members of the MAP/ERK pathway.²⁶ All of these results suggest a role for PBR in 1 if not several cellular pathways that regulate cell proliferation, differentiation, or apoptosis. To better understand PBRs role in the progression of cancer and to begin to realize PBRs cancer therapeutic and prognostic potential, more in depth studies of PBRs ability to either regulate or to be regulated by each of these pathways are necessary.

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Short Communication

**Peripheral-type Benzodiazepine Receptor (PBR) Gene
Amplification in MDA-MB-231 Aggressive Breast Cancer Cells**

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Abbreviated title: PBR gene amplification in breast cancer cells

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Summary

Recent studies using human breast cancer cell lines, animal models, and human tissue biopsies have suggested a close correlation between the expression of the peripheral-type benzodiazepine receptor (PBR) and the progression of breast cancer. This study investigates the genetic status of the PBR gene in two human breast cancer cell lines: MDA-MB-231 cells, which are an aggressive breast cancer cell line that contains high levels of PBR, and MCF-7 cells, which are a non-aggressive cell line that contains low levels of PBR. Both DNA (Southern) blot and fluorescence *in situ* hybridization (FISH) analyses indicate that the PBR gene is amplified in MDA-MB-231 relative to MCF-7 cells. These data suggest that PBR gene amplification may be an important indicator of breast cancer progression.

Introduction

The peripheral-type benzodiazepine receptor (PBR) is an 18 kDa high affinity cholesterol-binding protein [1,2] involved in numerous biological functions, including steroid biosynthesis, mitochondrial respiration, and cell proliferation [3,4]. PBR is present in most tissues examined [3,4] and upregulated in various tumors and carcinomas, such as gliomas [5,6], ovarian carcinomas [7], colon adenocarcinomas [8], endometrial carcinomas [9] and hepatomas [10]. Recent studies demonstrated that PBR is upregulated in both aggressive human breast cancer cell lines [11,12] and in metastatic human breast tumors [11] compared to non-aggressive tumor cells [11,12] and normal breast tissue [11]. The nature of this upregulation in breast cancer and other tumors is unknown.

Gene amplification is a well-known mechanism in tumorigenesis. Several well-known oncogenes are found amplified in various cancers such as *erbB-2* in breast cancer and *N-myc* in neuroblastomas (reviewed by Savelyeva and Schwab [13]) and the *AIB1* gene in breast and ovarian cancers [14]. In order to determine if the increases in PBR expression seen in aggressive breast cancer are due to amplification of the PBR gene, we examined the PBR gene in the aggressive MDA-MB-231 and the non-aggressive MCF-7 human breast cancer cell lines using both Southern blot and fluorescence *in situ* hybridization (FISH) analyses.

Materials and Methods

Cell Culture The MDA-MB-231 and MCF-7 cell lines, obtained from the Lombardi Cancer Center, Georgetown University Medical Center, were grown in DMEM, 10% FBS as previously described [11]. Cell growth curves for MDA-MB-231 and MCF-7 cells were established by Crystal Violet staining of the cells as we previously described [15].

Southern blot analysis Genomic DNA from each cell line was isolated using the QIAamp tissue kit (Qiagen, Inc.). 20 µg of each DNA sample was digested with *EcoR I* restriction enzyme, electrophoresed on a 1% agarose gel and transferred to derivatized nylon membranes (Nytran Plus, Schleicher & Schuell, Inc.) [16]. The -317 to +36 bp fragment of the PBR gene was radiolabeled with [α-32P]dCTP using a random primer DNA labeling system (InVitrogen, Inc.). The membrane was first prehybridized overnight at 68°C in 6X SSC, 0.5% SDS and 100 µg/ml denatured, fragmented, salmon sperm DNA. After hybridization, the membrane was washed twice with 2X SSC, 0.5%

SDS, for 10 min, once with 0.2X SSC, 0.5% SDS, for 30-60 min at room temperature, and once with 0.2X SSC, 0.5% SDS, for 30 min at 60°C. Screen-enhanced autoradiography was performed by exposing the blots to BioMax MS (Kodak, Rochester, NY) film at -80°C for 4 to 48 hours. Densitometric analysis of the spots was performed using the SigmaGel software (Jandel Scientific, San Rafael, CA).

FISH Genomic BAC clone dJ526I14 containing a 129 kb fragment of human chromosome 22 known to contain the PBR gene was obtained from Sanger Molecular Labs, UK. Presence of the PBR gene was verified by DNA (Southern) blot using PBR cDNA as probe (data not shown). BAC DNA was purified using the Qiagen Large Construct Kit and preliminary characterization of the PBR gene was performed using gene specific primers and fluorescent automated DNA sequencing. FISH analysis confirmed the correct chromosomal location of the BAC clone on chromosome 22q13.31 (data not shown). Using nick translation, the isolated DNA was labeled with biotin-16-dUTP (Boehringer Mannheim Corporation) and hybridized to interphase nuclei prepared from the cells as described previously [17]. The Biotin-labeled DNA was detected using Fluorescein-Avidin DCS (FITC) (Vector Lab Inc.). The nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) staining. Over 200 interphase nuclei were counted for each cell line using a Leica DMRBE fluorescent microscope.

Results

In agreement with previously published reports, MDA-MB-231 cells grow very fast whereas MCF-7 cells, in the absence of exogenously added estrogen, grow very slowly in culture (Figure 1). The possibility that PBR overexpression in aggressive

human breast cancer cells is due to PBR gene amplification was investigated by both DNA (Southern) blot (Figure 2) and FISH analyses (Figure 3). Southern blot analysis indicated that the aggressive MDA-MB-231 cells contain higher (4-fold) levels of PBR gene compared to MCF-7 cells. FISH analysis further confirmed these results. Counting over 200 cells from each cell line showed that 32% of the MDA-MB-231 cells have 3 or more copies of the PBR gene compared to only 4% of the MCF-7 cells (Table I).

Discussion

This study used both FISH and Southern blot analyses to demonstrate amplification of the PBR gene in MDA-MB-231 cells, which are an aggressive, estrogen receptor (ER) negative human breast cancer cell line. Conversely, MCF-7 cells, which are a non-aggressive, ER positive human breast cancer cell line, did not demonstrate amplification of the PBR gene. The *in vitro* growth parameters, invasive and metastatic behavior of the cells used in the present study as well as their *in vivo* tumorigenicity in nude mice has been previously reported [11,18]. In agreement with these studies, we observed that MDA-MB-231 cells grow at a much faster rate than the MCF-7 cells *in vitro* and grew tumors when implanted in SCID mice [15]. In contrast, MCF-7 cells did not grow tumors in this animal model system (Hardwick and Papadopoulos, unpublished data). FISH evaluation showed an increased copy number of the PBR gene in over 30% of the cells in MDA-MB-231 compared to only 4% of MCF-7 cells. The FISH assay does not rule out other aberrations of chromosome 22. Southern blot analysis indicated that MDA-MB-231 cells contain higher (4-fold) levels of PBR gene compared to MCF-7 cells.

We have recently shown that PBR levels are greatly increased in metastatic breast tumors compared to normal mammary epithelium [11]. Further, we showed that PBR protein, mRNA, and specific ligand binding is increased in aggressive breast cancer cell lines relative to non-aggressive breast cancer cell lines [11]. This correlation was most readily seen between the MDA-MB-231 and MCF-7 cell lines and has subsequently been confirmed by others [12]. We also showed that the ability of MDA-MB-231 cells to form tumors *in vivo* might depend on the amount of PBR present in the cells [15]. At the conclusion of these studies we discussed the possibility that these increases may be due either to amplification of the PBR gene and/or increased transcription/translation of the gene in aggressive tumors relative to non-aggressive tumors and normal tissue [11].

This study suggests that increases in PBR expression seen in previous studies may be at least in part due to amplification of the PBR gene. However, gene amplification does not appear to be solely responsible for the increases seen in PBR expression due to the fact that the PBR gene is increased 4-fold and PBR mRNA is increased at least 20-fold in MDA-MB-231 cells relative to MCF-7 cells [11]. Therefore, it appears that the increase in PBR expression in more aggressive cancers is the result of both gene amplification and increased gene transcription/translation.

Given the upregulation of PBR in tumors and the correlation between increased PBR expression and the aggressive character of breast cancer cell lines, future studies will consider whether PBR expression can function as a prognostic indicator of the progression of specific cancers. Presently, we do not know whether the findings presented in this study represent the *in vivo* situation in human biopsies or if they reflect a peculiarity of the cell lines used. We are currently investigating whether PBR gene

amplification also occurs in human biopsies and whether this amplification could be used as a breast cancer prognostic indicator.

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Table I. FISH Analysis of MDA-MB-231 and MCF-7 Human Breast Cancer Cell Lines.

Over 200 interphase nuclei were isolated from both MDA-MB-231 and MCF-7 cells. Nuclei were labeled with a BAC fragment containing a portion of the PBR gene as described in Materials and Methods. Nuclei were subsequently counted for the presence of 1, 2, or 3 or more signals.

| Cells | FISH Signals | | |
|-------------------|------------------------|--------------------------|-----------------------|
| | 1 | 2 | 3 or more |
| MDA-MB-231 | 3 of 214 cells (1%) | 143 of 214 cells(67%) | 68 of 214 cells (32%) |
| MCF-7 | 16 of 210 cells (7.5%) | 186 of 210 cells (88.5%) | 8 of 210 cells (4%) |

Figure Legends

Figure 1. MDA-MB-231 and MCF-7 cell proliferation rates. Cells were plated at a density of 2,000 cells per well on 96 well plates and followed for 5 days. Every 24 hours, cells were stained with Crystal Violet. Absorbance was read at 570nm. The results shown are means \pm S.D. (n=8) representative of 3-4 independent experiments.

Figure 2. DNA (Southern) blot analysis of PBR in MCF-7 and MDA-MB-231 (MDA-231) cells. Genomic DNA was digested with *EcoR I* and hybridized to PBR full-length cDNA probe as described under Materials and Methods.

Figure 3. FISH analysis of PBR gene in MCF-7 and MDA-MB-231 (MDA-231) cells. DNA from BAC clone dJ526I14 (Sanger Molecular Labs, UK) was labeled with biotin-16-dUTP and hybridized to interphase nuclei prepared from the 2 cell lines MCF-7 and MDA-MB-231 as described under Materials and Methods. The Biotin-labeled DNA was detected using fluorescein-avidin DCS (FITC). MCF-7 cells showing 2 FISH signals and MDA-MB-231 cells showing 3 signals are shown.

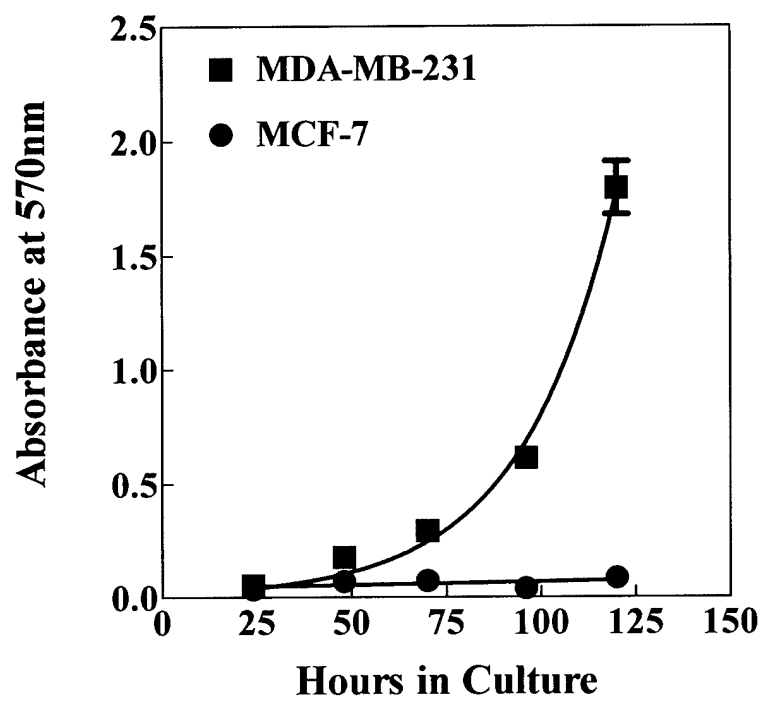


Figure 1

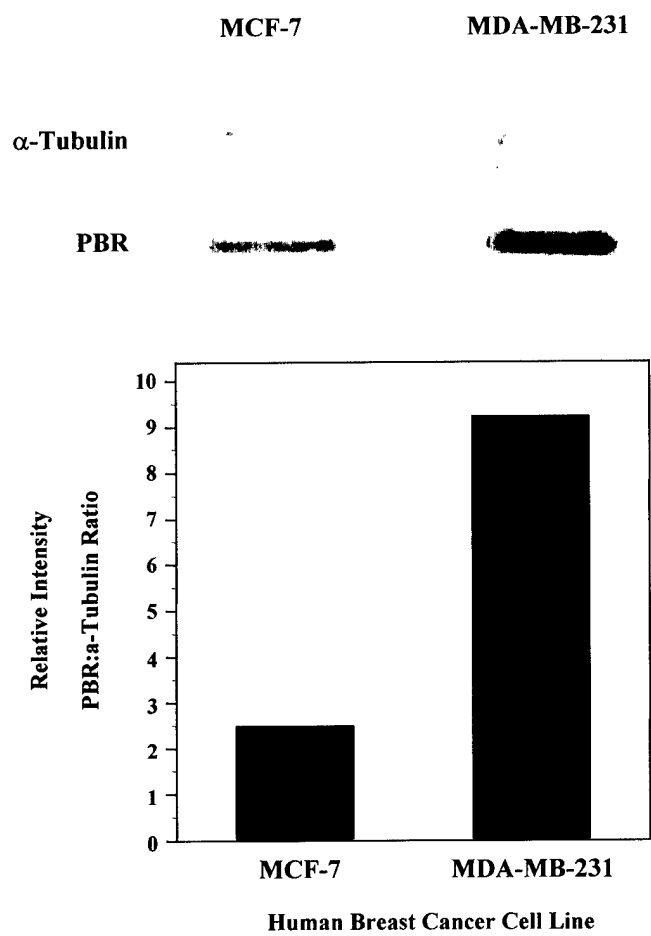


Figure 2

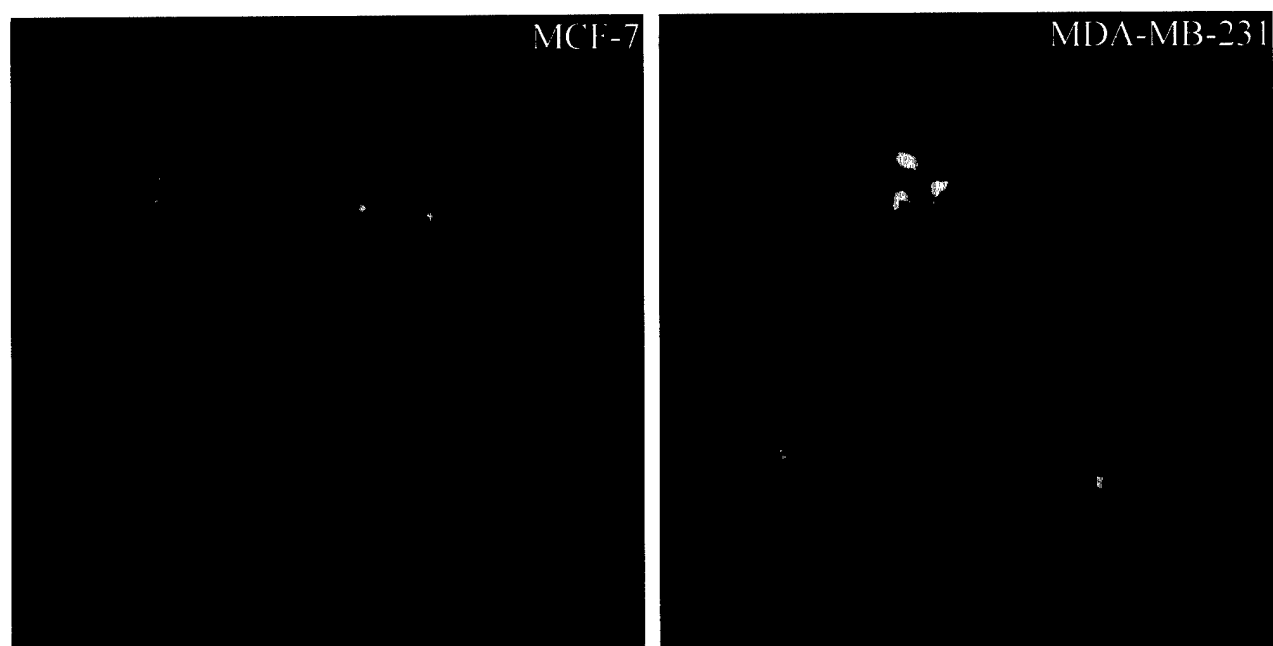


Figure 3